Indonesian Journal of Biotechnology, December (1997) 168 - 177

Development of DNA recombinant vaccine for type 1 bovine viral diarrhea virus *in vitro*

R. Wasito[1] and Hastari Wuryastuti[2]

1) Department of Pathology, Faculty of Veterinary Medicine/Inter University Center for Biotechnology, Gadjah Mada University, Yogyakarta 55281, Indonesia

2) Department of Internal Medicine, Faculty of Veterinary Medicine/Inter University Center for Food and Nutrition, Gadjah Mada University, Yogyakarta 55281, Indonesia

Abstract

A DNA recombinant bovine viral diarrhea virus (BVDV) vaccine based on adenovirus vector has been developed for this purpose. The recombinant adenovirus type 5 (Ad5) contains a deletion of a portion of the E1 region. In the present study, the foreign genes for BVDV of type 1 those are the 53 Kd glycoprotein (gp53) and 53 Kd glycoprotein with signal peptide sequence (gp53s) were inserted into adenovirus genome vector, the Ad5 strain within the E1 region. In this case, the gp53 and gp53s of BVDV were expressed by the Ad5 vector in 293 cell line of human embryonic kidney, in which Ad5 replication is efficient. It was concluded that E1-deleted Ad5 is suitable for expressing BVDV gene(s) and has the potential advantage of being used as a general approach to a DNA recombinant vaccination of BVDV.

In addition, the researchs on the incidence of BVDV infection in Indonesian cattle using immunoperoxidase monolayer assay (IPMA) have recently been developed and performed in the Laboratory of Immunochemistry/Chemistry, Inter University

Indonesia. In addressing the global needs for effective control of BVDV in Indonesia, additional experiment(s) in the use of Ad5 vector in the DNA recombinant vaccine production of NCP-BVDV of Indonesian (local) isolates are being developed.

Keywords: *Recombinant adenovirus type 5 – type 1 BVDV strain – gp53-gp53s – human embryonic kidney cell*

Introduction

Bovine viral diarrheal virus (BVDV) is the prototype member of the genus Pestivirus. Pestiviruses were initially classified in the family Togaviridae, but were recently transferred to the family Flaviridae because the genetic organization of the pestivirus genome and its encoded polypeptides are similar to flaviviruses (Collett *et al.*, 1989). Two other viruses of importance comprise the pestiviruses: hog cholera virus and border disease virus of sheep.

The genome of BVDV consists of a single positive-strand RNA of approximately 12.5 Kb. A single large open reading frame (ORF) spans the length of the genome and codes for approximately 4,000 amino acids (Collett *et al.*, 1989). The virus encoded proteins of the ORF have been identified, their coding regions have been localized, and the entire polypeptide complement has been elucidated (Collett *et al.*, 1991).

BVDV was first recognized and reported

from North America in 1946, in association with epizootics of acute and often fatal disease, characterized by diarrhea and erosive lesions of the digestive tract (Olafson *et al.*, 1946).

In Indonesia, in one study, serological prevalence of antibody evidence to BVDV in cattle on rural farms was reported. A total of 413 cattle sera (198 of sera from Holstein Friesian crossbred cattle and 215 of sera from Ongole crossbred cattle) were examined for anti-BVDV antibodies. The prevalence in Holstein Friesian crossbred cattle (35.9%) is far above that of Ongole crossbred cattle (6.5%) (Wasito *et al.*, 1992).

Recent research efforts have focused on defining the pathogenesis of MD (McClurkin *et al.*, 1984; Dufell and Harkness, 1985; McClurkin *et al.*, 1985; Baker, 1987; Brownlie, 1990; Bolin *et al.*, 1991). To understand the pathogenesis it is first necessary to introduce the concept of BVDV biotypes. Two biotypes of BVDV are recognized, cytopathic (CP) and non-cytopathic (NCP) which are used to classify BVDV isolates based on their behavior in tissue culture (Donis and Dubovi, 1987). Although the origin of the CP-BVDV can be external, as evidenced by documented outbreaks of MD following the use of modified-live-virus vaccines (Baker, 1987), current theory holds that in the majority of cases of MD the CP-BVDV represents a mutation of the NCP-BVDV (the persistent infecting biotype). Following mutation, the occurrence of MD is dependent upon the antigenic relationship between the CP and NCP-BVDV, with homologous antigenic relationship believed to result in MD. Molecular studies on BVDV support the theory that there is a mutational event in which the NCP-BVDV mutates to a CP-BVDV. Evidence is accumulating that the mutational event that induces a switch in biotype is an insertion of cellular RNA within the p125 gene region (Qi *et al.*, 1992). Nature p80 gene product is only found in cytopathic strain and results from proteolytic cleavage of the p125 precursor.

In the present study, it was proposed to develop the capacity of a recombinant adenoviral vector to produce gp53 and gp53s genes of BVDV expression. This approach to Ad-1 vectored vaccines may be adapted for future DNA recombinant vaccines against BVDV of Indonesian isolates.

Materials and Methods

Cell culture and virus propagation

The type 1 BVDV strain was propagated in bovine turbinate (BT) cells as described earlier (Wasito and Hastari,1996). This isolate belongs to the cytopathic biotype. The BT cells were grown in Eagle's Minimal Essential Medium (EMEM) (Gibco, Grand Island, N.Y.) supplemented with 10% fetal equine sera (FES) (Gibco-BRL, Grand Island, NY, USA), 100 IU/ml penicillin G (Sigma Chemical Co, Dekalb, St. Louis, MO, USA), 100 ug/ml streptomycin (Sigma Chemical Co, Dekalb, St. Louis, MO, USA), 1 ug/ml fungizone (Gibco-BRL, Grand Island, NY, USA) and 1% L glutamine 200mM (Gibco-BRL, Grand Island, NY, USA) and sodium bicarbonate to adjust the pH to 7.4. The BT cells were free of adventitious BVDV by IPMA (Wasito and Wuryastuti, 1996; Wuryastuti and Wasito, 1996). The type 1 BVDV strain was allowed to adsorb to the BT cells for 1 h before the maintenance medium was added. Under these conditions, the cytopathic effect reached 80-90% on the third day post infection.

Nucleic acid extraction

The BT cells of cultures showing a cytopathic effect of about 80-90% were directly scraped in the culture flask. After centrifugation at 200 rpm, the supernatant was

poured off and cells pellet was treated with Tri Reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA). Viral RNA extraction and isolation by the Tri Reagent DNA/RNA protein isolation method was performed as described previously (Wuryastuti and Wasito, 1997).

cDNA synthesis, reverse-transcriptase polymerase chain reaction (RT-PCR) amplification and cloning

Primers used for cDNA synthesis and RT-PCR amplification are presented in Figure 1. Aliquots representing 5% of the total RNA extracted from infected BT cell cultured grown in 75 cm^2 flasks were used for cDNA synthesis, followed by RT-PCR amplification. Reverse transcription of RNA (Promega Corporation, Madison, WI, USA) and RT-PCR amplification of cDNA were done using a kit (Gene Amp PCR Core Reagent, Perkin-Elmer, Norwalk, CT., USA), following the manufacturer's directions. The amplification reaction consisted of 45 cycles of 94° C for 1 min, 50°C for 1 min, 72°C for 1 min. Amplified gp53 and gp53s products were cloned into the pT7/T3α-19 plasmid vector (Gibco-BRL, Grand Island, NY, USA) (Fig. 2) and subcloned into pCA14 and pHCMVsp1D plasmid vectors (Fig. 3).

Primer 1: 5'-TAGGATCCACCATGAGGGGCCAGGTGGTACAG 32-mer
Primer 2: 5'-TAGGATCCACCATGCACTTGGATTGCAAACCTGAAT 36-mer
Primer 3: 5'-TTCCCGGGTTATTCTGATAAGACCATGTATGT 32-mer

Figure 1. Primers 1 (86; 2406 genomic location), 2 (87; 2463 genomic location) and 3 (88; 3580 genomic location) used for amplification of BVDV gp53 and gp53s genes RT-PCR.

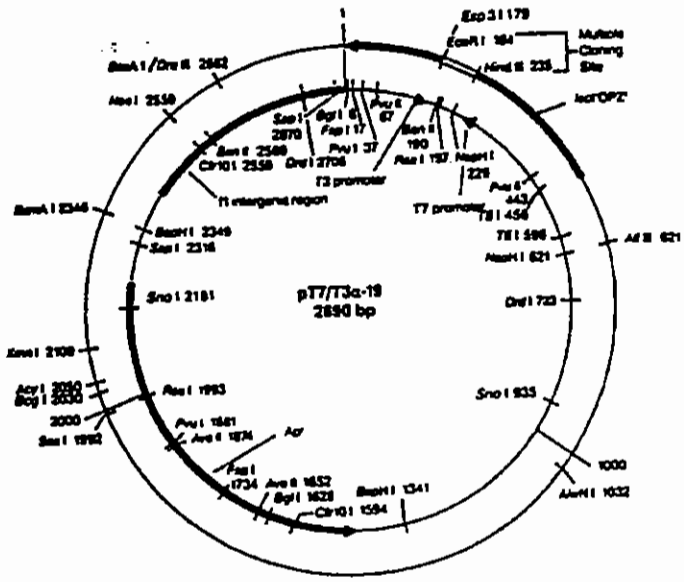


Figure 2. A map of pT7/3α-19 plasmid vector. A vector used to introduce gp53 and gp53s genes into Ad5 DNA E_1 region insertion plasmid vectors of pCA14 or pHCMVspID.

Cotransfection of human embryonic kidney cells (293 cells) with pCA14gp53, pHCMVspIDgp53, pCA14gp53s or pHCMVspIDgp53s with pJM17

The 293 cells were grown in a 60 mm dish containing Dulbecco's Minimal Essential Medium (DMEM) (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine sera (FBS), 1% glutamine and 1% penicillin G+streptomycin overnight. After 24 hours incubation at 37° C in 5% CO_2 incubator, the medium was removed from the 293 cells. The cells were washed twice with PBS. The DNA used for cotransfection was prepared from pCA14gp53+pJM17 and pHCMVsp1Dgp53+pJM17, pCA14gp53s+pJM17 and pHCMVsp1Dgp53s+ pJM17 (Fig. 4). A pFG140 plasmid was used as the positif control plasmid for recombination with 293 human embryonic kidney cells (Fig. 5). Cotransfection was accomplished by adding

DNA suspension to each dish and incubated in 5% CO_2 incubator at 37°C. The plaque or cytopathic effects were observed after 3-4 days.

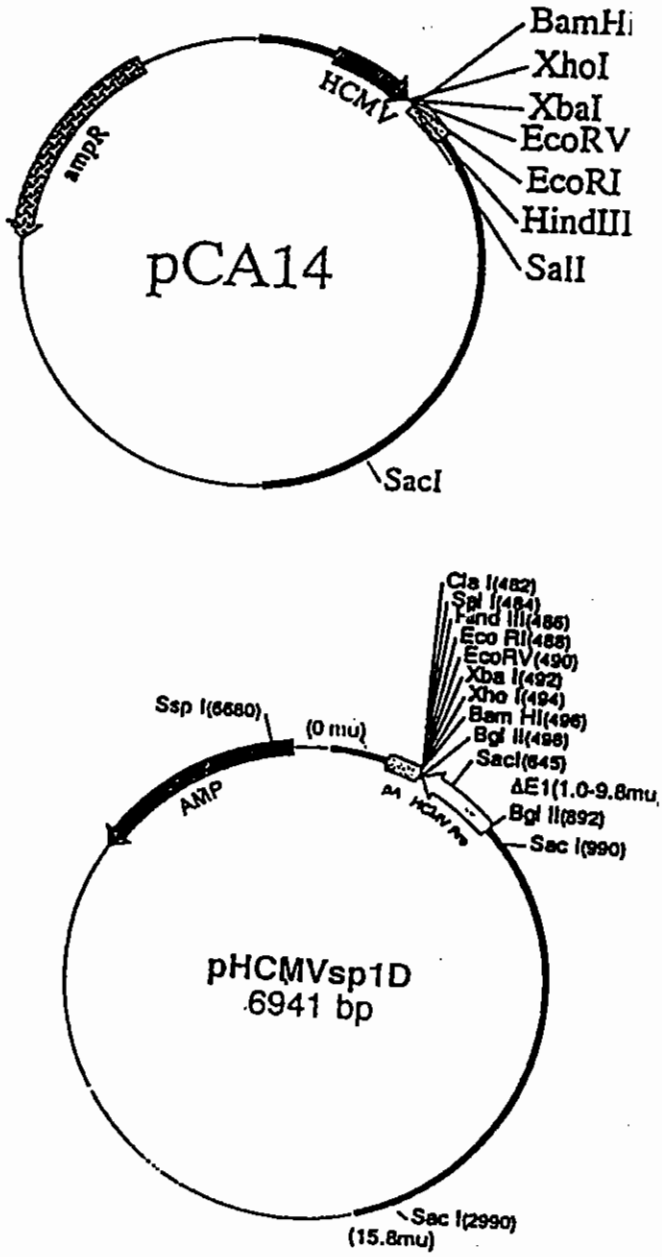


Figure 3. A map of Ad5 DNA E_1 region insertion of pCA14 and pHCMV-spID plasmid vectors. Vectors used to introduce gp53 and gp53s genes insert into Ad5 DNA E_1 region from rescue plasmid pJM17.

Cotransfection of human embryonic kidney cells (293 cells) with pCA14gp53, pHCMVspIDgp53, pCA14gp53s or pHCMVspIDgp53s with pJM17

The 293 cells were grown in a 60 mm dish containing Dulbecco's Minimal Essential Medium (DMEM) (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine sera (FBS), 1% glutamine and 1% penicillin G+streptomycin overnight. After 24 hours incubation at 37° C in 5% CO_2 incubator, the medium was removed from the 293 cells. The cells were washed twice with PBS. The DNA used for cotransfection was prepared from pCA14gp53+pJM17 and pHCMVsp1Dgp53+pJM17, pCA14gp53s+ pJM17 and pHCMVsp1Dgp53s+ pJM17 (Fig. 4). A pFG140 plasmid was used as the positif control plasmid for recombination with 293 human embryonic kidney cells (Fig. 5). Cotransfection was accomplished by adding DNA suspension to each dish and incubated in 5% CO_2 incubator at 37° C. The plaque or cytopathic effects were observed after 3-4 days.

Results and Discussions

Adenoviruses which have been used extensively as a model system for molecular study of mammalian cell DNA replication, transcription and RNA processing are now being increasingly investigated as a potential mammalian expression vectors for gene therapy and for recombinant vaccines (Berkner, 1988). There are many reasons for this renewed popularity of Ad vectors: the 36,000 bp double stranded DNA genome of Ad is relatively easy to manipulate by recombinant DNA techniques, the genome does not undergo rearrangement at a high rate, the viral particle is relatively stable and the adenovirus replicates efficiently in permissive cells, producing up to 10,000 plaque

forming units (PFU) per infected cells, thus enabling the production of high titer viral stocks. Late in infection, most of the infected cell protein is viraly encoded, potentiating the use of replication-proficient recombinant Ads as short term-high level expression vectors. In nondividing nonpermissive cells the viral genome may persist as an episome and continue to express for long periods. Finally, a variety of different cell types can be transformed by integration of Ad DNA in the host cell genome. If the efficiency of this can be increased, Ad recombinant may also be useful for gene transfer into mammalian cel chromosome. The long-term goal of the present study is to develop a DNA recombinant technology for producing DNA recombinant vaccine of BVDV Indonesian isolates. A first step towards the development of this DNA recombinant technology was to create an *in vitro* system to test the formation of DNA-plasmids complexes and their ability to deliver DNA to 293 human embryonic kidney cell line by cotransformation.

Replication competent adenoviruses are readily generated by the transfection of adenovirus DNA into susceptible cells. Furthermore, adenovirus subgenomic fragments have been cloned in various bacterial plasmids. The desired recombinant can be made by inserting the gene of interest into one such fragment which is transfected with a complementing fragment into a mam-

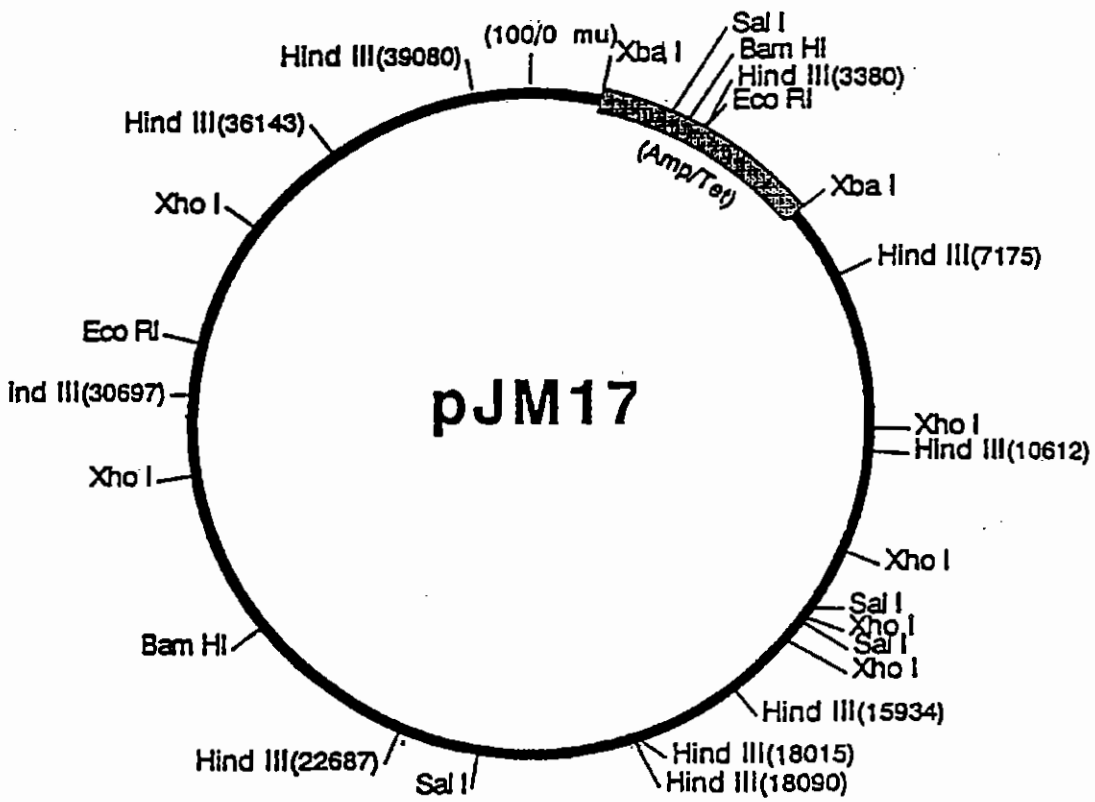


Figure 4. A map of Ad5 DNA E_1 region of pJM17 rescue plasmid vector. The recombinant plasmids of pCA14gp53, pCA14gp53s, pHCMVspIDgp53 or pHCMVgp53s were cotransfected with the pJM17 into 293 human embryonic kidney cells.

malian cell where homologous recombinant can occur. In this study, the foreign genes for BVDV of type 1 strain or the 53 Kd glycoprotein (gp53) and 53 Kd glycoprotein with signal peptide sequence (gp53s) were inserted into adenovirus genome vector, the Ad5 strain within the E1 region (Figs. 6 and 7). In this case, the E1 region is deleted to allow for a larger insert. Although, insertions in the E1 region usually result in a replication defective virus, this virus can be grown on cells, such as the 293 cell line of human embryonic kidney (Graham *et al.*, 1977). This 293 cell line has been stably transfected with functional $E1_a$ and $E1_b$ genes that complement the defect of $E1_a/E1_b$ deletions in the virus itself.

The Ad5 strain used in this study was selected on the basis of previous studies indicating its safety in humans (Schwartz *et al.*, 1974). The Ad5 vector that was initially utilized to overproduce proteins made in small amounts during various viral infections (Berkner *et al.*, 1987; Davidson and Hassell, 1987; Mansour *et al.*, 1986), are now showing significant permissive delivering gp53 or gp53s genes for the production of DNA recombinant vaccine of BVDV. Previous works have reported that the Ad5 virion has the ability to express proteins for DNA recombinant vaccine against hepatitis B virus (HBV) (Levrero *et al.*, 1991), human immunodeficiency virus-1 (HIV-1) (Prevec *et al.*, 1991), herpesvirus (Marshall *et al.*, 1990; Eliot *et al.*, 1990), rhabdovirus (vesicular stomatitis virus and and rabies) (Schneider *et al.*, 1989; Prevec *et al.*, 1989) and respiratory syncytial virus (RSV) (Hse *et al.*, 1991).

Results of the cell culture in this study demonstrated that the gp53 and gp53s of BVDV was expressed by the Ad5 vector in 293 cell line of human embryonic kidney, in

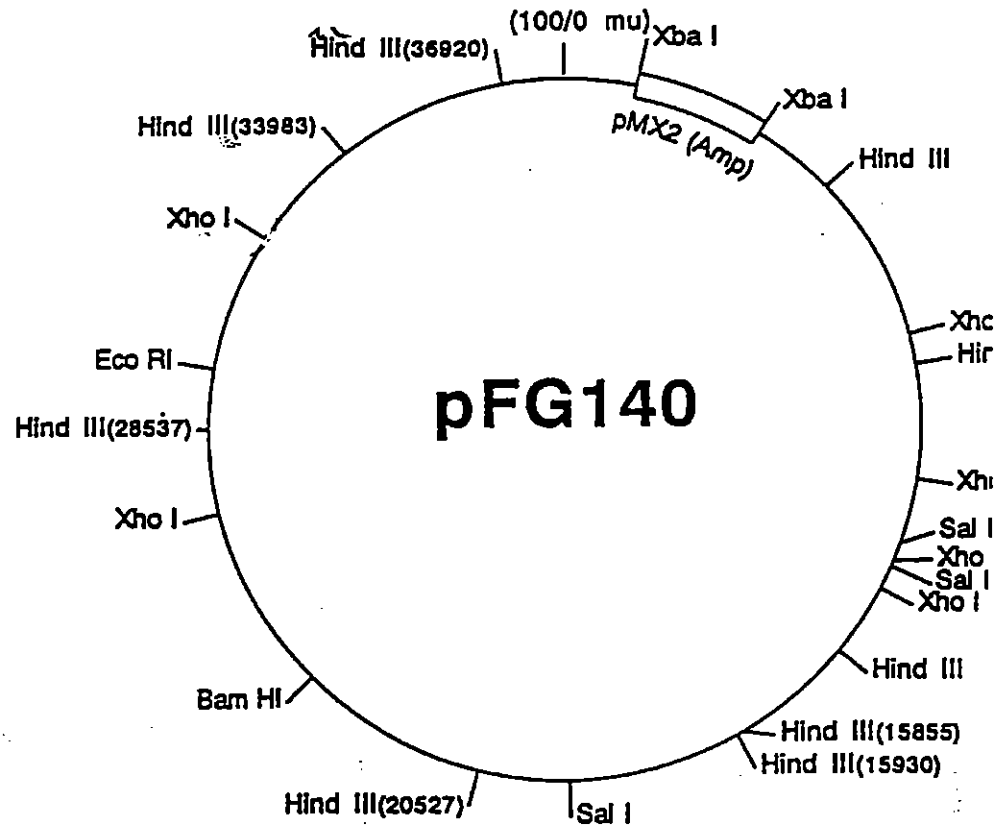
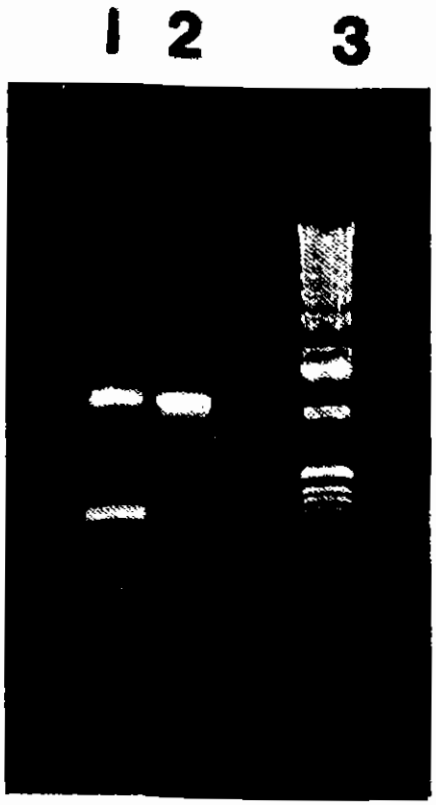


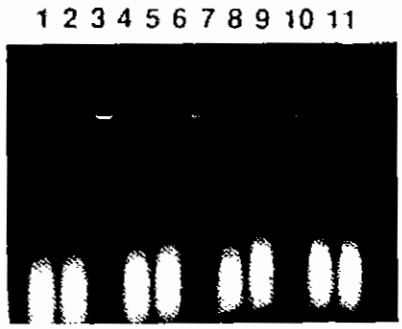
Figure 5. A map of pFG140 plasmid used as the positif control plasmid for recombination with 293 human embryonic kidney cells.

which Ad5 replication is efficient which is only after short incubation times (72-96 hours). This study is not entirely completed yet. Additional testing, include simplicity of preparation, efficacy and ability to induce a sufficient, good immune response will be of considerable interest in terms of possible development of adenoviral vector vaccines and in the study of the immune response to BVDV of Indonesian isolates.



Lane 1: gp53
Lane 2: gp53s
Lane 3: Ladder marker DNA 1 Kb

Figure 6. Gel electrophoresis analysis of gp53 and gp53s genes. The genes were resolved by 0.6% agarose gel and stained with ethidium bromide.



Lane 1: Plasmid pCA14gp53s
Lane 2: Plasmid HCMVsp1Dgp53s
Lane 3: Plasmid pCA14gp53 (Wizard)
Lane 4: Plasmid pCA14gp53s
Lane 5: Plasmid pHCMVsp1Dgp53s
Lane 6: Plasmid pHCMVsp1Dgp53 (Wizard)
Lane 7: Plasmid pCA14gp53s
Lane 8: Plasmid pHCMVsp1Dgp53s
Lane 9: Plasmid pHCMVsp1Dgp53 (Wizard)
Lane 10: Plasmid pCA14gp53s
Lane 11: Plasmid pHCMVsp1Dgp53s

Figure 7. Gel electrophoresis analysis of plasmids pCA14 and pHCM-VspID ligated with each gp53s and gp53 genes. The genes were resolved by 0.6% agarose gel and stained with ethidium bromide.

Conclusion

It has been demonstrated that the Ad5 virus is capable of generating gp53 or gp53s in 293 cell line of human embryonic kidney cell. This approach to Ad5-vector foreign genes expression may be adapted for development and production of DNA recombinant vaccine of BVDV Indonesian isolates.

Acknowledgment

This work was supported by Biotechnology Career Fellowships of the Rockefeller

Foundation and Michigan Agriculture Experimental Station, Large Animal Clinical Science, Department of Microbiology and Animal Health Diagnostic Laboratory, College of Veterinary Medicine, Michigan State University, USA.

The authors wish to thank Prof. Dr. Roger K. Maes, as the adviser, Dr. Ir. Joedoro Soedarsono, Director of the Inter-University Center for Biotechnology, Gadjah Mada University, Yogyakarta, Indonesia for his thoughtful advise, Dr. Aivars Vilnis, Dr. Michael Sussmann, Mr. Daniel Taylor, Mrs. Alice Murphy and Ms. Lori Kunze for their technical assistance, Dr. John C. Baker, Large Animal Clinical Sciences, Michigan State University, USA. for useful discussions.

References

Baker, J.C. (1987) Bovine viral diarrhea virus: A review. *J.A.V.M.A.* 190: 1449-1458.

Berkner, K.L. (1988) Development of adenovirus vectors for expression of heterologous gene. Biotechniques 6: 616-629.

Berkner, K.L., Schaffhausen, B.S., Roberts, T.M. and Sharp, P.A. (1987) Abundant expression of polyoma virus middle T antigen and dihydrofolate reductase in an adenovirus recombinant. *J. Virol.* 61: 1213-1220.

Bolin, S.R., Matthews, P.J. and Ridpath, J.F. (1991) Methods for detection and frequency of contamination of fetal calf serum with bovine viral diarrhea virus and antibodies against bovine viral diarrhea virus. *J. Vet. diagn. Invest.* 3: 199-203.

Brownlie, J. (1990) Pathogenesis of mucosal disease and molecular aspects of bovine viral diarrhoea virus. *Vet. Microbiol.* 23: 371-382.

Collett, M.S., Moenning, V. and Horzinek, M.C. (1989) Recent advances in pestivirus research. *J. Gen. Virol.* 70: 253-266.

Collett, M.S., Wiskerchen, M.A., Welniak, E. and Belzer, S.K. (1991) Bovine viral diarrhea virus genomic organization. *Arch. Virol. Suppl.* 3: 19-27.

Davidson, D. and Hassell, J.A. (1987) Overproduction of polyomavirus middle T antigen in mammalian cells through the use of an adenovirus vector. *J. Virol.* 61: 1226-1239.

Donis, R.O. and Dubovi, E.J. (1987) Differences in virus-induced polypeptides in cells infected by cytopathic and noncytopathic biotypes of bovine virus diarrhea-mucosal disease virus. *Virology* 158: 168-173.

Dufell, S.J. and Harkness, J.W. (1985) Bovine virus diarrhea-mucosal disease infection in cattle. *Vet. Rec.* 117: 240-245.

Eliot, M., Gilardi-Hebenstreit, P., Toma, B. and Perricaudet, M. (1990) Construction of a defective adenovirus vector expressing the pseudorabies virus glycoprotein gp50 and its use as a live vaccine. *J. Gen. Virol.* 71: 1785-1792.

Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36: 9-72.

Hse, K.H.L., Lubeck, M.D., Davis, A.R., Bhat, R.A., Selling, B.H., Bhat, B.M., Mizutani, S. and hung, P.P. (1991) Immunogenicity and protective efficacy of adenovirus vectored respiratory syncytial virus vaccine. In vaccines 91. Eds. Chanock, R.M., Ginsberg, H.S., Brown, F. and Lerner, R.A. *Cold Spring Harbor Laboratory Press.*: 293.

Levrero, M., Barban, V., Manteca, S., Ballay, A., Balsamo, C., Avantaggiati, M.L., Natoli, G., Skellekens, H., Tiollais, P. and Perricaudet, M. (1991) Defective and nondefective adenovirus vectors for expressing foreign genes *in vitro* and *in vivo*. *Gene* 101: 195-202.

Mansour, S.L., Grodzicker, T. and Tjian, R. (1986) Downstream sequences affect transcription initiation from the adenovirus major late promoter. *Mol. Cell Biol.* 6: 2684-2694.

Marshall, G.S., Ricciardi, R.P., Rando, R.F., Puck, J., Ge, U., Plotkin, S.A. and Gonczol, E. (1990) An adenovirus recombinant that expresses the human cytomegalovirus major envelope glycoprotein and induces neutralizing antibodies. *J. Infect. Dis.* 162: 1177-1181.

McClurkin, A.W., Bolin, S.R. and Coria, M.F. (1985) Isolation of cytopathic and noncytopathic bovine viral diarrhea virus from the spleen of cattle acutely and chronically affected with bovine viral diarrhea. *J. Am. Vet. Med. Assoc.* 186: 568-569.

McClurkin, A.W., Littledike, E.T. and Cutlip, R.C. (1984) Production of cattle immunotolerant to bovine viral diarrhea virus. Can. *J. Comp. Med.* 48: 156-161.

Olafson, P., MacCallum, A.D., Fox, F.H. *et al.* (1946) An apparently new transmissible disease of cattle. *Cornell Vet.* 36: 205-213.

Prevec, L., Christie, B.S., Laurie, K.E., Bailey, M.M.S., Graham, F.L. and Rosenthal, K.L. (1991) Immune response to HIV-1 gag antigens induced by recombinant adenovirus vectors in mice and rhesus macaque monkeys. *J. Acq. Imm. Def. Syn.* 4: 568-576.

Prevec, L., Schneider, M., Rosenthal, K.L., Belbeck, L.W., Derbyshire, J.B. and Graham, F.L. (1989) Use of human adenovirus-based vectors for antigen expression in animals. *J. Gen. Virol.* 70: 429-434.

Qi, F., Ridpath, J.F., Lewis, T., Bolin, S.R. and Berry, E.S. (1992) Analysis of the bovine viral diarrhea virus genome for possible cellular insertions. Virology 189: 285-292.

Schwartz, A.R., Togo, Y., and Hornick, R.B. (1974) Clinical evaluation of live, oral types 1, 2 and 5 adenovirus vaccines. *Am. Rev. Respir. Dis.* 109: 233-238.

Schneider, M., Graham, F.L. and Prevec, L. (1989) Expression of the glycoprotein of vesicular stomatitis virus by infectious adenovirus vectors. *J. Gen. Virol.* 70: 417-427.

Wasito, R., Sardjono, B., Wuryastuti, H., Isrina, S. and Yuriadi. (1992) Uji serologik *Bovine Viral Diarrhea* dengan teknik *Enzyme-Linked Immunosorbent Assay*. PAU Bioteknologi, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Wasito, R. and Wuryastuti, H. (1996) Repropagation, reisolation and reidentification of the Indonesian non-cytopathic bovine viral diarrhea virus isolates *in vitro*: Means of immunoperoxidase monolayer assay. *I. J. Biotech* June: 28-35.

Wuryastuti, H and Wasito, R. (1996) Detection of bovine viral diarrhea virus (BVDV) in milk samples by immunoperoxidase monolayer assay. *I. J. Biotech* December: 66-69.

Wuryastuti, H and Wasito, R. (1997) Application of the polymerase chain reaction technique for detecting bovine viral diarrhea virus (BVDV) in bulk milk samples. *I. J. Biotech* June: 113-116.